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Biofilms and infections of the upper respiratory tract

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Abstract. – Biofilms are microbial communities consisting of bacteria that either are self-reproducing on biological surfaces or are present in the lifeless environment. Biofilms are quite diffuse entities frequently found in human pathological conditions. The formation of bacterial biofilms involves mainly the contamination of artificial medical devices, such as valves and catheters, and their direct implant on mucous membranes, with subsequent development of chronic or recurrent infections.

Bacterial biofilms show a complex organization consisting of bacterial cells adherent to a surface and surrounded by a large extracellular matrix mostly made up of polysaccharides and proteins.

The resistance observed in biofilms does not appear to be genotypic; instead, it is due to multicellular strategies and/or to the ability of each cell, contained inside the biofilm, to differentiate into a protected phenotypic state which tolerates the antibiotic action. In fact, biofilms are subject to changes following their recurrent exposure to antimicrobial agents, thus incrementing their resistance.

Biofilms play an important role in otitis media, sinusitis, chronic cholesteatomatous otitis media, tonsillitis and adenoiditis, thus demonstrating that adenoidectomy may be helpful to children suffering from such a morbid conditions.

It is presently estimated that biofilm formation is involved in at least 60% of all chronic and/or recurrent infections. In addition, 30% of the exudates developing in the course of otitis media has shown to be positive for the presence of biofilms; likewise biofilms have been found in tonsillar crypts and in odontostomatologic infections as well.

Studies have been carried out on both the use and the efficacy of N-acetylcysteine (NAC) in biofilm breakdown. It has been shown that NAC, used at different concentrations, is able to reduce bacterial adhesion in several anatomical districts.

Key Words:

Biofilms, Upper Respiratory Infections (URI), N-acetylcysteine (NAC).

Introduction

Biofilms are microbial communities consisting of bacteria that either are self reproducing on biological surfaces or are present in the lifeless environment. Biofilms are quite diffuse entities frequently found in human pathological conditions. From an environmental standpoint, biofilm formation is involved mostly in contamination and corrosion processes. Any surface, which is in frequent or continuous contact with water, becomes covered, within a short time, by a film composed of bacterial biofilms, that is subject to changes in its characteristics and properties.

In human pathology, bacterial biofilm formation involves mainly the contamination of artificial medical devices, such as valves and catheters, as well as their direct implant on mucous membranes, leading to the development of chronic or recurrent infections. It is currently estimated that biofilm formation is involved in at least 60% of chronic and/or recurrent infections.

Resistance observed in biofilms appears not to be genotypic, that is induced by plasmids and trasposons, nor consequent to mutational events; instead, it is rather due to multicellular strategies and/or to the ability of individual cells, contained inside the biofilm, to differentiate in a protected phenotypic state which tolerates antibiotic action.

Groups of well characterized mutant strains have shown to be useful means aimed at searching the mechanisms by which bacteria realize biofilm formation.

As far as *Escherichia coli* is concerned, mutant isolates have been obtained through mutagenesis by trasposons insertion. Such strains were unable to produce type I pils or were poorly motile. Type I pils are proteins belonging to the mannose-sensitive adhesin family and their role in the initial stages of biofilm formation has been substantiated by experiments in which a mannose analogue inhibited biofilm formation by the mutant strain. The

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importance of motility is due to the fact that the flagellar absence, or the flagellar paralysis, in mutant bacteria leads to a reduced ability to generate biofilms. The small number of hypomotile cells, that were able to adhere to polyvinylchloride (PVC), remained assembled in sparse groups. Once the surface is reached, type I pils adhere thus generating a cell-surface adhesion. Then cell motility promotes biofilm diffusion over the surface.

As far as *Pseudomonas aeruginosa* is concerned, bacterial strains unable to start biofilm formation on PVC showed some deficit either in flagella-mediated motility or in type IV pils biogenesis. Mutants without type IV pils formed scattered single layers; they were unable to develop either any assembled single layer or any bacterial microcolony. Retraction and diffusion of type IV pils induced cell migration through a surface, thus generating a contracting motility, designated as "twitching". Relevant to *Pseudomonas aeruginosa*, it seems that both flagella and flagella-mediated motility are important with respect to adhesion and formation of single dispersed cell layers. Type IV pils, instead, play an important role in the production of confluent pellicles and in the accumulation of the cells into microcolonies.

All that has been mentioned above shows clearly how cellular structures, such as flagella, pils and other surface proteins, play important roles in the initial phases of biofilm formation. Furthermore, the various mentioned structures play distinct roles according to bacterial species and to the different environmental conditions as well.

Biofilm formation and their subsequent development occur according to three main phases:

Adhesion: bacteria start to fasten to organic and to inorganic surfaces, following initially a reversible fashion;

Colonization and microcolonies formation: intercellular adhesion occurs during the second phase and it reflects the starting point for the realization of biofilms complex structure. During this stage gene transcription occurs, which is essential for the production of extracellular polysaccharides. In the absence of this process, genes would be repressed;

Maturation: this process requires the presence of particular communicating signals, that are able to regulate, inside the biofilm, distribution of individual bacterial species, protein expression in the surrounding cells and gene expression.

Following their entrapment inside the exopolysaccharide (EPS) matrix, bacteria return with some difficulty into a planktonic shape, that is into a condition fluctuating in a liquid. However, their detachment from the biofilm may occur because of either modified environmental conditions or modifications in the substrate. Consequently, released bacteria behave as planktonic entities and may induce symptoms of clinical exacerbation and/or may initiate a new biofilm in locations that are far away from the starting point.

Bacteria gather huge benefits from biofilm formation; in fact, such benefits include a protection from environmental changes relevant to humidity, temperature, pH and ultraviolet ray exposure, as well as a concentrated amount of nutritional substances together with an easy excretion of waste products.

Inside the biofilms, close proximity of bacteria facilitates the development of intercellular interactions. Biofilm formation favors bacterial resistance towards defense mechanisms of the host, since bacterial aggregation into the EPS matrix originates structures that are too voluminous to undergo phagocytosis and reduces bacterial vulnerability towards the humoral immune system.

At the fourth Conference on Biofilms, held in 2007 in Canada, the relationship between bacteria and human host has been analyzed in two sections. *In vivo* images were shown of bacterial biofilms during infections, using a combination of *in situ* fluorescence of antibodies and steel, in order to bring into evidence biofilms associated with infected sutures or with lining epithelia of upper respiratory tract mucosal membranes (adenoids and middle ear). In several instances the patients showed symptoms of bacterial infection even though cultures were negative[1].

Analyses performed on pediatric adenoid tissue allowed for the formulation of the four criteria categorizing biofilms:

- Pathogenous bacteria are attached to the surface or are adherent to a substrate;
- Direct examination shows clusters of bacteria as well as bacteria contained into a bacterial matrix or into host's components;
- Localized infection; and
- The infection is resistant to antibiotic therapy.

The first three criteria were established on the basis of microscopic observations. The fourth criterion was supported by empirical evidence of unsuccessful pharmacological treatment.

A large proliferation of biofilms has been found in four test-tubes of tympanotomy obtained from children suffering from persistent ot-

orrhea. The presence of biofilms was observed on tympanic membranes also during clinical remissions of the disease[2].

Furthermore, the presence of biofilms in 94.9% of the adenoid surface of children suffering from rhinosinusitis and/or chronic otitis, as compared with 1.9% of the surface of children submitted to adenoidectomy for obstructive apnea, suggests a correlation between the effectiveness of the surgical removal of pharyngeal tonsils and the detachmnet of the adhesive surface of the biofilm[3].

Biofilm-Induced Chronic Otitis Media

Chronic otitis media is an example in which the focalization upon the bacterial component leads to an imperfect understanding of the pathophysiology of biofilm formation. In the vast majority of instances of acute otitis media, exudates show positive cultures, whereas exudates from chronic otitis media are positive only in 30% of the cases[4]. During the past decade, pediatric otolaryngologists of Pennsylvania (USA), by using a combination of molecular diagnosis and imaging studies conducted in humans and in laboratory animals, have demonstrated that effusive otitis media (EOM) associated with negative cultures and otorrhea, is actually an active bacterial infection due to the presence of a biofilm on the middle ear mucous membrane. The study carried out by such physicians shows that, although a variety of inflammatory mediators are present in chronic otitis media, the phlogistic process is guided by the bacterial biofilm located on the middle ear mucous membrane[5-8].

The direct findings of biofilms in bioptic specimens of middle ear mucous membranes of children with EOM or recurrent otitis media supports the hypothesis that the symptomatology linked to chronic otitis media may be biofilm-induced[9].

Roveta et al[10], Rise et al[11] have demonstrated that N-acetylcysteine (NAC), in doses equivalent to those employed in clinical practice[5-20] mg/ml), is capable of reducing bacterial adhesion to oropharyngeal epithelial cells *in vitro* (Figure 1). According to Rise et al[11], the decreased adhesion contributes to explain the reduction in infectious episodes following the aerosol administration of NAC.

Biofilm-Induced Chronic Tonsillitis

A study performed at Washington University of St. Louis (USA)[12] has evidenced the presence of biofilms inside the crypts in 1 out of 15 tonsils with chronic tonsillitis and in 3 out of 4 hypertrophic tonsils. The biofilms were made up of gram-negative and gram-positive bacteria and by extracellular polymeric substances. Therefore, the Authors concluded that a strong anatomic evidence exists pertinent to the presence of bacterial biofilms in tonsils presenting with chronic infections; as a consequence, the chronic course and the recurrent pattern of certain forms of tonsillitis may be explained.

A study issued in 2008[13] has evidenced that biofilms were present il 85% of tonsils removed on account of a chronic infection, in 41% of tonsils removed for eliminating an obstruction, and in 5% of tonsils removed because they were both infected and obstructive. In such a study the possible presence of biofilms on the adenoids was

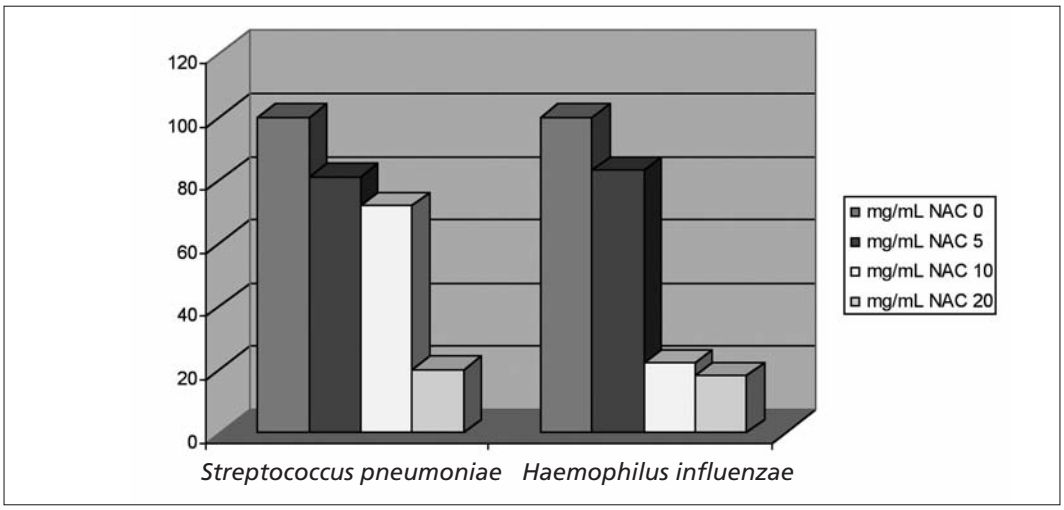

Figure 1. N-acetylcysteine action on bacterial adhesiveness in an epithelial cell model. N-acetylcysteine action on bacterial adhesiveness in a model of oropharyngeal epithelial cells.

also taken into consideration, and biofilms were actually found in 67% of the specimens.

In patients with infection and recurrent obstruction, gram-negative and gram-positive bacteria were found; *Haemophilus influenzae* also played a significant role[14].

Biofilms and Cholesteatoma

Persistent and recurrent otorrhea is a clinical feature of cholesteatoma, which is very hard to eradicate by means of antibiotics and usually responds only to surgical removal. Areas of gram-positive and gram-negative bacteria, enclosed into the biofilm polysaccharide matrix, have been found in cholesteatoma keratin[15]. The Authors of the study have concluded that a strong morphologic evidence exists *in situ* with respect to the existence of biofilms inside cholesteatomas. Moreover, the presence of biofilms provides an explanation for the numerous clinical characteristics of infected cholesteatomas.

While biofilms of bacterial nature are extensively described in literature, very little is known about biofilms of fungal origin. Recent experimental evidence has shown the way in which catheter infections due to *Candida* species are associated with biofilm production.

Biofilm-associated fungal infections are often resistant to conventional therapy because of the development of a highly resistant phenotype, in all likelihood consequential to an over-regulation. For such a reason, the new classes of antifungal agents, such as the amphotericin lipidic formulations and the echinocandins, that show a typical activity against resistant biofilms produced by *Candida* species, may aim to treating invasive systemic mycosis.

Recently, the etio-pathogenetic importance of *Trichosporon asahii* has been emphasized; such a micro-organism is an opportunistic fungal pathogen emerging mostly in neutropenic and immunodepressed patients. *Trichosporon asahii* shows a marked adhesive capacity towards polystyrene since it colonizes the surface within 30 min of incubation. The amount of biofilm produced increases exponentially following the initial 24 hours and reaches its maximum amount in 72 hours. In general, the biofilm produced by *Trichosporon asahii* is resistant to the *in vitro* action exerted by fluconazole, voriconazole, amphotericin B and caspofungin, although it shows different degrees of resistance[16]. Azoles fungistatic action favors genetic adaptation that leads to the formation of drug-resistant clinical isolates involving several mechanisms, that range from the change in drug uptake to the modification in permeability and to the increase in the efflux pump.

Several Authors have pointed out that the ability of fungal pathogens to adhere to surfaces and to form biofilms is strongly correlated with morphogenesis, virulence and resistance towards antifungal drugs. The results achieved have shown morphologic changes, including increase in dimensions, alteration in septal formation and in cellular wall organization together with a different distribution of both the mannanic and the glucanic components of the most external layer of the cellular wall.

Significant advances in the field of implantable medical devices realized in the past few decades must be ascribed in part to the growing development of new and more suitable synthetical materials (Figure 2). However, their use may still be associated with serious infectious complications. Several responsible microbial species are encoun-

Biofilm formation inside an urinary catheter

Biofilm-induced corrosive phenomena

Dental plaque

Figure 2. Biofilms in the environment and in human pathological conditions.

tered, among which the most common are the gram-positive bacteria (*staphylococci*) and the yeasts (*Candida*). The best experimental polymer/antibiotic system realized up to date consists of a basic polymer (polyurethan) treated with an acidic antibiotic (rifampicin); such a system has shown to be capable of inhibiting *Staphylococcus epidermidis* colonization for an 8-month period.

Biliary stent implants by endoscopic route represent a therapeutic approach increasingly used for the treatment of biliary tract pathologic conditions. Microbiological investigations carried out up to now have evidenced the growth of fungal species (*Candida* spp.), aerobic and anaerobic bacterial species (*Escherichia coli, Enterobacter, Enterococcus, Klebsiella, Bacteroides, Clostridium*). On the other hand, scanning electronic microscopy observations have substantiated the presence of coccoid and bacillar forms together with fungal forms in sessile growth, as shown by the National Institutes of Health[18] (Table I).

In the odontostomatologic field, it has been observed that *Helicobacter pylori*, a gastroduodenal pathogen, is able to survive under unfavorable environmental conditions through a non cultivable status (VBNC), resembling the population of persisters, which constitutes that portion of heterogeneous biofilms responsible for the marked antibiotic resistance. The presence of cells assembled in large clusters embedded into an abundant matrix has been evidenced following two days of incubation of motionless sessile cultures[19].

The wide use of contact lenses has urged industrial and medical research on identifying new biomaterials with a better tolerability. A medical problem linked to contact lenses use derives from their sensitivity towards adhesion and colonization by biofilm-producing bacteria.

Table I. List of infections involving biofilms.

Dental caries	Gram-positive cocci
Periodontitis	anaerobic gram-negative bacteria
Otitis media	*Haemophilus influenzae*
Necrotizing fascitis	group A streptococci
Biliary tract infections	*Escherichia coli*
Musculoskeletal infections	gram-positive cocci
Osteomyelitis	Mixed bacterial and fungal conditions
Prostatitis	*E. coli* and gram-negative bacteria

Chemicophysical characteristics of biomaterials used for the production of contact lenses may influence biofilm formation. In the present study we have investigated whether some difference in bacterial colonization exists between two types of contact lenses: one of them is realized by means of a phosphorylcholine-containing material (Proclear, CooperVision, Inc., Irvine, CA, USA) while the other type contains Balafilcon A (Pure Vision, Bausch & Lomb, Rochester, NY, USA).

Data obtained from MIC and MBC tests have shown that sensitivity to antibiotics is greater in those bacteria that had colonized on phosphorylcholine-containing contact lenses. Such results suggest that phosphorylcholine-containing contact lenses may reduce the risk of bacterial keratitis due to *Pseudomonas aeruginosa*[20] (Table II).

A study has been published on biofilm formation on firm surfaces[21]. The Authors have pointed out that the initial adhesion is dependent upon the substrate humidity.

The growth of various bacterial species, both in monocultures and in multispecies communities culture medium, is inhibited by a number of NAC concentrations[22].

Table II. Biofilms contaminating medical devices and materials.

Contaminated devices	Biofilms (main bacterial species)
Contact lenses	Gram-positive cocci and *Pseudomonas aeruginosa*
Devices for peritoneal dialysis	Mixed bacterial and fungal flora
Urinary catheters	*Escherichia coli* and other gram-negative bacteria
IUD	*Actinomyces israeliti*
Endotracheal devices	Mixed bacterial and fungal flora
Venous catheters	*Staphylococcus epidermidis*
Mechanical cardiac valves	*Staphylococcus aureus* and *Staphylococcus epidermidis*
Vascular implants	Gram-positive cocci
Orthopedic devices	*Staphylococcus aureus* and *Staphylococcus epidermidis*
Prostheses for a number of organs	*Staphylococcus aureus* and *Staphylococcus epidermidis*

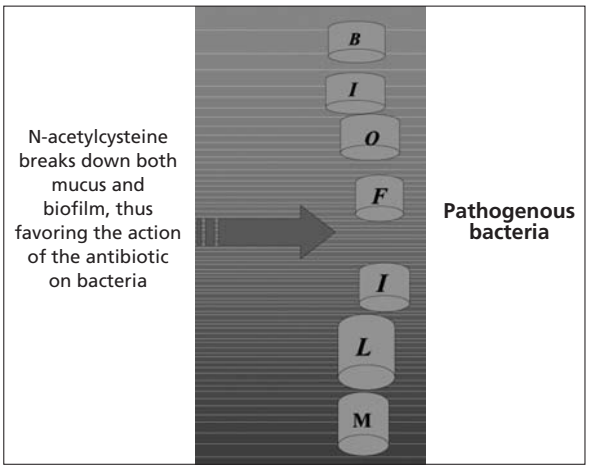

Figure 3.

In addition, it has been demonstrated that NAC has not broken down extracellular polysaccharides. Moreover, NAC presence modifies the structure generated by the biofilm; this makes NAC an interesting candidate to be utilized for the inhibition of bacterial biofilm formation on steel surfaces (Figure 3).

Even certain chemical substances, such as EDTA and NAC, are able to promote the break down of preformed *Pseudomonas aeruginosa* biofilms[23]. Some studies have recently demonstrated NAC inhibitory effect on slime synthesis carried on by *Staphylococcus epidermidis*[24].

NAC is not an antibiotic drug but it possesses antibacterial properties; in addition it is endowed with both a strong mucolytic activity, which is capable of decreasing the viscosity of secretions, thus facilitating bacterial elimination from the respiratory tract[25], and an inhibiting activity on bacterial adhesiveness to respiratory tract epithelial cells[11].

As it is possible to detect in the study performed on *Staphylococcus aureus* strains, NAC has failed to induce visible changes in bacterial growth even at the maximum concentration tested (8 mg/ml). In all strains, however, NAC has inhibited slime production. At the 8 mg/ml concentration, biofilm reduction over 50% (namely 68.2%, 56.3% and 58.9%) were observed in 3 strains (*Staphylococcus* 1393, 1890 and 1880, respectively)[11] (Figure 4).

In the report by Schito and Marchese published in 2006[26] the mechanisms supporting NAC bacterial action have been analyzed and the spectrum of its direct antimicrobial activity has been evaluated with respect to the most important pathogenous agents responsible for respiratory infections, as well as to the oral cavity microorganisms and to some anaerobic bacteria. Ultimately, NAC *in vitro* interactions with several classes of antibiotics were assessed. NAC showed to be a bactericidal drug exerting a concentration-dependent activity.

In addition, NAC has shown to be a molecule which is highly capable of influencing bacterial viability in a negative way, as evaluated on the basis of colony forming units per milliliter (CFU/ml), with a 63% mean reduction in biofilm cells at an initial maturation stage and a 52%

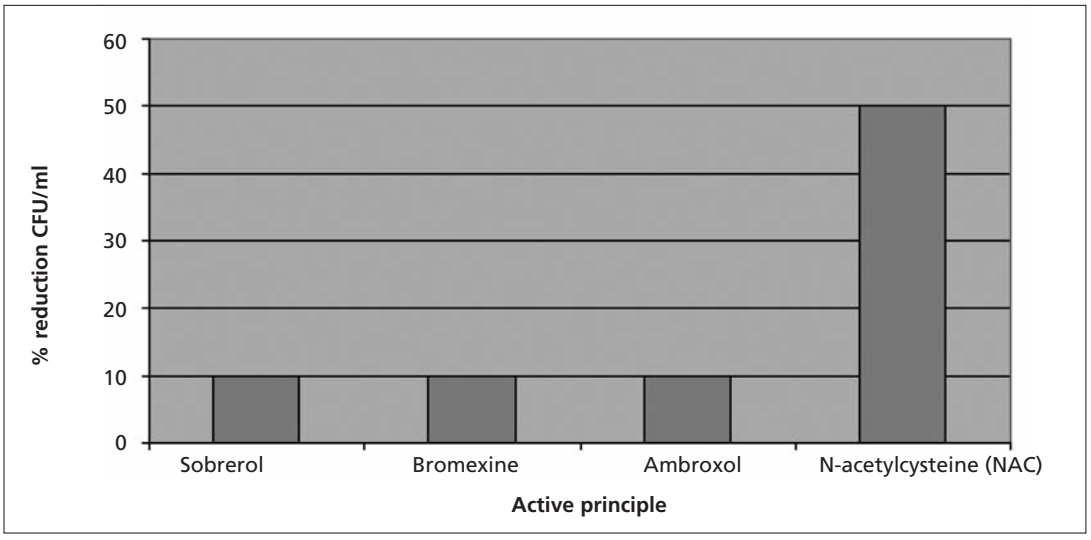

Figure 4. Effects of muco-active agents (8 mg/ml) on viability of biofilms produced by *Staphylococcus aureus*. Comparison between sobrerol, bromexine, ambroxol and N-acetylcysteine[11].

mean reduction of biofilm cells at a fully consolidated maturation stage[26].

Ambroxol has shown a 12% mean reduction in biofilms at initial maturation and 11% on those at consolidated maturation, sobrerol 13% and 11%, respectively, and bromexine 12% and 10%, respectively.

Among the numerous presently available muco-active drugs administered by aerosol, only NAC is endowed with a true direct mucolytic activity; such a substance is also able to induce some inhibition and degradation of biofilms and to influence in a negative way the viability of sessile bacterial populations[27].

With respect to *Staphylococcus aureus* 1876, biofilm production has shown a 30.3% reduction[22]. The mean value was found to be 51%. At the maximum concentration used, NAC has broken down biofilms produced after 5 hours of bacterial growth (initial maturation) for over a 50% value (namely 61.1%, 57.3% and 58.6%) (mean degradation 52.4%) in 3 strains (1393, 1880 and 1890, respectively). Relevant to *Staphylococcus aureus* 1876, the calculated reduction was 32.5%. NAC reduced the amount of mucopolysaccharidic material of biofilms with fully consolidated maturation over 50% in all the 4 strains tested (62%, 60.5%, 58.1% and 65.5%) (mean degradation 61.5%). At the maximum concentration used, NAC has brought about a reduction in bacterial viability on biofilm cells both at the initial maturation stage and at the consolidated maturation stage. Such a reduction was measured on the basis of colony forming units per ml (CFU/ml) and was found to vary from 71.2% to 91% (mean reduction 83.2%) with respect to cells belonging to the youngest biofilms and from 33.8% to 70.6% (mean reduction 46.3%) for the cells of mature biofilms. Lower NAC concentrations brought about reductions in viable cells varying from 46.8% to 0% (immature biofilms) and from 11.7% to 0% (mature biofilms)[26].

The sensitivity of tested microorganisms has shown to be markedly variable both between and within the species: gram-positive pathogens have shown to be more sensitive. However, gram-negative bacteria also were influenced by the antimicrobial effect, although they required higher NAC concentrations.

A greater inhibiting activity of the molecule has been observed against *Streptococcus pneumoniae* and *Staphylococcus pyogenes* as compared to other respiratory and oral gram-positive bacteria. *Moraxella catarrhalis* and *Haemophilus influenzae* have shown a greater *in vitro* sensitivity towards NAC as compared to other gram-negative bacteria[28].

When used in association with antibiotics of different categories, NAC, in the presence of certain molecules, such as amoxicillin/clavulanic acid, cefaclor, cefditoren, thiamphenicol, fosfomycin, claritromycin and levofloxacin, has shown additive effects on aerobic, anaerobic, gram-positive and gram-negative oral or respiratory bacteria. A bactericidal synergism has been evidenced in certain strains of *Staphylococcus pyogenes* and *Streptococcus pneumoniae* when NAC was associated with amoxicillin/clavulanic acid, cefaclor and cefditoren[29].

In an Authors' preliminary study, two groups of pediatric patients, suffering from recurrent upper respiratory tract infections, were enrolled. The first group was treated with NAC by the oral route for a 6-month period; the second group did not receive NAC. The results of the study evidenced a reduction in phlogistic episodes in the NAC-treated group.

On the basis of the results obtained in such a study, it is presumed that the clinical use of NAC, as a topical antimicrobial drug, can be carried on; in such a context NAC must be taken into consideration as an useful additive component of classical antibiotic therapy, as it may produce a synergic additive or bactericidal effect on current etiologic pathogenous agents.

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